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Purification and Characterization of the Lethal Toxin (Alpha-Toxin) of *Clostridium septicum*

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***Clostridium septicum* lethal (alpha-toxin) was purified and found to be a basic protein (pI 8.4) of approximately 48 kDa that is both lethal and hemolytic. The alpha-toxin had a hemolytic activity of approximately 2 × 10^7 hemolytic units per mg and a 50% lethal dose of approximately 10 μg/kg of body weight for mice. The alpha-toxin formed concentration-dependent, sodium dodecyl sulfate-resistant aggregates of approximately 230 kDa. Mice immunized with alpha-toxin showed a significant increase in survival time over mock-immunized mice when challenged with *C. septicum*. Rabbit polyclonal antibody was generated against the purified toxin and was used to confirm that toxin with the same molecular weight was present in seven different *C. septicum* isolates. No proteins in the supernatants from cultures of *Clostridium perfringens*, *Clostridium histolyticum*, *Clostridium chauvoei*, or *Clostridium difficile* were found to react with the *C. septicum* alpha-toxin-specific antibody.**

Historically, *Clostridium septicum* has played a significant role as a causative agent of traumatic gas gangrene. However, in recent years, *C. septicum* has become increasingly identified with nontraumatic gas gangrene in patients with various diseases that affect the colon (1, 4, 5, 10–12, 14, 18, 25). Generally, patients that present with a *C. septicum* infection are often found to have a colonic carcinoma or a tumor that has metastasized to the colon. Other patients at risk of this disease include leukemia patients, diabetics, or patients with cyclic neutropenia. Patients that develop *C. septicum* gangrene generally exhibit a poor prognosis due to the fulminant nature of the disease and the fact that early diagnosis is difficult. Without early treatment, the mortality rate is near 100% (12).

The toxins of *C. septicum* have not been studied extensively, and none have been purified (that we know of [19]). Four 'toxins' have been shown to be separate entities by either serologic studies or enzymatic activity. These include the lethal and necrotizing toxin (alpha-toxin), DNase (beta-toxin), hyaluronidase (gamma-toxin), and the thiol-activated toxin or septicolysin (delta-toxin) (19). Other enzymes, such as protease and neuraminidase, are also produced by *C. septicum* (6). Two hemolytic activities have been described for *C. septicum*. One is presumably a thiol-activated cytolysin, and the other is the lethal toxin or alpha-toxin (7, 21). The alpha-toxin of *C. septicum* is not related to the alpha-toxin of *Clostridium perfringens* since it does not exhibit phospholipase C activity and does not cross-react immunologically with *C. perfringens* alpha-toxin (reviewed in reference 19). In the present study, we purified the lethal toxin of *C. septicum* and determined some of its characteristics.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. *C. septicum* BX96 (2) (a generous gift from A. Bernheimer) was used for the purification of alpha-toxin. This strain was confirmed as *C. septicum* by biochemical analysis and gas chromatography of membrane lipids. The *C. septicum* strains and other clostridial species used in this study are described in Table 1.

All clostridial strains were cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 0.05% cysteine at 37°C for 18 to 24 h in an anaerobic hood (Forma Scientific, Marietta, Ohio). Large-scale growth of *C. septicum* was initiated by inoculating two 3-liter containers of sterile brain heart infusion broth (with 0.05% cysteine) with a 1:100 inoculum from an overnight culture of *C. septicum* BX96. The large cultures were incubated as described above. All chemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified.

Lethality assay. To identify the lethal fractions during the purification, we injected mice (Swiss Webster outbred females, 18 to 24 g) intraperitoneally with 100 μl of the desired fraction and monitored time to death. Generally, several fractions were pooled to decrease the number of assays required. Time to death was monitored for each mouse to obtain an estimate of the most lethal fractions.

A 50% lethal dose (LD_{50}) for the purified lethal toxin (alpha-toxin) was determined as follows. Mice (four Swiss Webster outbred females per group) were injected intraperitoneally with serially diluted alpha-toxin in sterile saline in a total volume of 200 μl. The numbers of survivors and mortalities were recorded for each group after 48 h. The LD_{50} was calculated by the method of Reed and Muench (17).

Hemolytic assay. The amount of toxin giving 50% hemolysis was estimated by making serial dilutions of the sample in phosphate-buffered saline (PBS; 10 mM $NaHPO_4$ [pH 7.0], 5 mM KCl, 145 mM NaCl, 0.1% glucose) and visually determining 50% hemolysis by the button size of the unlysed erythrocytes. The sample (5 to 50 μl) was diluted into a final volume of 100 μl in the first well of a 96-well microtiter plate, and to the remaining wells, 50 μl of PBS was added. Serial 1:2 dilutions were performed by removing 50 μl of the first dilution, adding it to 50 μl of PBS in a subsequent well, and mixing thoroughly. This was repeated until 8 to 12 dilutions had been made. To each well, 50 μl of an erythrocyte

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TABLE 1. Sources of the various clostridial species

Clostridial species	Strain	Source
C. septicum	BX96	A. Bernheimer (2)
C. septicum	CS-23	A. Bernheimer (2)
C. septicum	25 (patient isolate)	Veterans Hospital, Oklahoma City, Okla. (16)
C. septicum	87-016055 (patient isolate)	Centers for Disease Control (3)
C. septicum	8065	ATCC[a]
C. septicum	11424	ATCC
C. septicum	12464	ATCC
C. perfringens (type A)	13124	ATCC
C. difficile	10463	Lyerly et al. (15)
C. chauvoei	10092	ATCC
C. histolyticum	19401	ATCC

[a] ATCC, American Type Culture Collection, Rockville, Md.

solution ($\approx 10^8$ washed human erythrocytes per ml) was added, and the plate was incubated at 37°C for 18 h. The hemolytic titer was taken as the last well to visually exhibit ≈50% hemolysis by the size of the button formed by the remaining unlysed erythrocytes.

Purification of alpha-toxin. All fractions were kept on ice except where indicated, and the chromatography was done at 4°C except for the Superose 12 and MA7S columns, which were run at room temperature. Approximately 6 liters of spent brain heart infusion broth from two 3-liter cultures of *C. septicum* (grown anaerobically for 18 h) was separated from the cells by centrifugation. The toxin-containing medium was then concentrated by using a Filtron tangential flow concentrator (Pharmacia, Piscataway, N.J.) equipped with 10,000-Da-cutoff membranes. The medium was concentrated to approximately 150 ml and then pressure dialyzed against 1 liter of 10 mM Tris-HCl (pH 8.0) (buffer A) using the same apparatus. This material was pumped (2 ml/min) over a column (3 by 20 cm) packed with the anion-exchange resin Accell QMA (Millipore Corp., Bedford, Mass.) equilibrated in buffer A. The unbound fraction containing the lethal activity was collected, concentrated by using a pressure cell (Amicon, Danvers, Mass.) equipped with a 10,000-Da-cutoff membrane (Millipore), and frozen at −20°C overnight. The next day the toxin solution was thawed and the pH was adjusted to 6.5 with HCl. The solution was allowed to sit on ice for at least 1 h until a precipitate formed. The precipitate was removed by centrifugation, and the solution was pumped onto a column (1.6 by 20 cm) packed with the cation-exchange resin Superose S (Pharmacia), which was equilibrated in 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid] (Research Organics, Cleveland, Ohio) (pH 6.5). The column was eluted (2 ml/min) with a 160-ml linear gradient from 0 to 0.5 M NaCl in the same buffer. The fractions exhibiting mouse lethality were concentrated to approximately 5 ml. The sample was chromatographed (1 ml/min) on a gel filtration column (1.6 by 50 cm) packed with preparative-grade Superose 12 (Pharmacia) equilibrated in MES buffer (pH 6.5) containing 100 mM NaCl. The active fractions were pooled and concentrated to approximately 5 ml. This material was loaded onto an MA7S high-resolution cation-exchange column (0.78 by 5 cm) (Bio-Rad Laboratories, Richmond, Calif.), and the protein was eluted (3 ml/min) with a gradient from 0 to 300 mM NaCl. The fractions exhibiting the majority of the lethality were pooled and concentrated to 1 to 2 mg/ml and made approximately 20% in glycerol. The protein was then stored at either −20 or −70°C. All high-resolution chromatography was performed with a titanium high-pressure liquid chromatography system and Dynamax software (Rainin Instruments, Woburn, Mass.).

Protein was assayed by a protein assay (Pierce Chemical Co., Rockford, Ill.) according to the manufacturer's instructions. Bovine serum albumin was used as the protein standard.

Rabbit polyclonal antibody production. Two New Zealand White rabbits were injected five times subcutaneously along the back and hip with 50 μg of pure lethal toxin in 300 μl of complete Freund's adjuvant. At 2 weeks, the animals were boosted with 50 μg of lethal toxin in 300 μl of incomplete Freund's adjuvant. Twenty-eight days following the initial immunization, the animals were bled by cardiac puncture, the blood was allowed to clot at room temperature for 1 h, and the serum was collected after centrifugation.

Gel electrophoresis and immunoblot procedures. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13), using a 10% resolving gel. All samples were diluted into sample buffer that contained 5% β-mercaptoethanol and then heated at 95°C for 2 min. For immunoblot analysis, proteins that were separated by SDS-PAGE were transferred to nitrocellulose by using a TE series Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.) according to the manufacturer's protocols. After transfer, the nitrocellulose paper was blocked with 3% nonfat dry milk in 10 mM Tris-HCl (pH 8.0)–100 mM NaCl–0.05% Tween 20. After 15 min, the primary antibody (anti-alpha-toxin) was diluted 1:1,000 in the blocking solution and added to the nitrocellulose blot. After an overnight incubation at room temperature, the blot was washed four times for 5 min each time with 100 ml of blot wash buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) to remove unbound antibody. The blot was then incubated for an additional 2 to 4 h with conjugate (goat anti-rabbit immunoglobulin G [IgG] conjugated with alkaline phosphatase) diluted 1:1,000 in blocking solution. The bands recognized by the primary antibody were visualized by using a solution (50 ml; 10 mM Tris, 10 mM NaCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, pH 9.5) containing 5-bromo-4-chloro-3-indoyl phosphate (6.5 mg) and nitroblue tetrazolium (5 mg). Development was stopped with a 5:5:1 mixture (vol/vol) of methanol, H_2O, and acetic acid.

Native acidic gel analysis of the purified toxin was performed by continuous acidic PAGE as described previously (8). A 10% resolving gel was used, and the gel and reservoir buffer was 0.05 M β-alanine, adjusted to pH 4.5 with acetic acid. The sample was prepared in the same buffer as for the gel and reservoir with the addition of glycerol to 10% and 0.002% methyl green as the tracking dye.

Affinity purification of antibody to the lethal toxin. For some experiments, it was necessary to affinity purify antibody that reacted only with the lethal toxin band. This was accomplished in the following way. The pure alpha-toxin was resolved in eight lanes of an SDS-polyacrylamide gel and transferred to nitrocellulose by the procedure described above. The nitrocellulose was incubated with the primary antibody, and a single lane was excised. The single-lane strip was then incubated in secondary antibody (goat anti-rabbit whole-molecule IgG conjugated with alkaline phosphatase). The strip containing secondary antibody was developed and aligned with the remaining seven lanes of the nitrocellulose to identify the location of the alpha-toxin band. The corresponding alpha-toxin region was excised from the immunoblot to give one nitrocellulose strip containing only the alpha-toxin band. This strip was then incubated for 5 min in pH 2.5 glycine to elute the bound antibody. The glycine-eluted antibody was neutralized immediately to ≈pH 7 with 50 μl of 1 M Tris-HCl (pH 8) per ml. The alpha-toxin-containing nitrocellulose strip was also neutralized with 20 mM Tris-HCl at pH 8. Following neutralization, the alpha-toxin-containing strip was incubated with a higher concentration of the antiserum (250 μl of antiserum in 1.75 ml of blocking solution) for 1 h. The process was repeated at least three times to provide a sufficient amount of antibody for use in an immunoblot analysis. The affinity-purified antibody solution from each elution was combined and concentrated, and fish gelatin was added to 1% to stabilize the antibody. This affinity-purified antibody was generally used to probe an immunoblot within 60 min of its purification. The same approach was used to affinity purify antibody to the aggregated alpha-toxin band.

Peptide mapping. Two-dimensional peptide mapping of the lethal toxin was accomplished as previously described (22). Briefly, purified lethal toxin was labeled with ^{125}I by using chloramine-T and then separated from excess ^{125}I by SDS-PAGE. The gel was stained with Coomassie R-250 dye and dried onto porous cellulose (Bio-Rad Laboratories), and the stained bands were excised. The dried gel slices were hydrated in a solution of 50 mM ammonium bicarbonate (pH 7.9) that contained 50 μg of TPCK (tosylamino-2-phenylethylchloromethylketone)-treated trypsin per ml. The gel slices were incubated in this solution for 18 to 24 h at 37°C. The supernatant fluid, which contained the ^{125}I-labeled tryptic peptides, was separated from the gel fragments and lyophilized. The pellet was resuspended in electrophoresis buffer (acetic acid-formic acid-H_2O, 3:1:16) to which 2 μl of a 0.1% solution of pyronine Y dye was added. The labeled peptides (1×10^6 to 2×10^6 cpm) were spotted onto the corner of a sheet (20 by 20 cm) of Kodak chromogram cellulose, and the plate was wetted with electrophoresis buffer. The tryptic peptides were separated by electrophoresis at a constant 500 V at 5°C on an FBE-3000 flatbed electrophoresis system (Pharmacia). The plate was dried and turned 90 degrees, and the peptides were chromatographed in a mixture of 1-butanol–pyridine–acetic acid–H_2O (32.5:25:5:37.5). Peptide spots were visualized following exposure of the chromatogram to X-ray film for 24 to 36 h at −70°C.

Mouse protection experiments. Mice were chosen for protection studies since it had previously been found that mice and guinea pigs were the most sensitive to *C. septicum* infection (19). Five groups of 10 Swiss Webster outbred mice were injected with 0.2 μg of lethal toxin (immunized group) in adjuvant (Ribi ImmunoChem Research, Hamilton, Mont.), and five groups of 10 Swiss Webster outbred mice were injected with normal saline and adjuvant (mock-immunized group). The lethal toxin was then frozen at −70°C and thawed as needed for booster immunizations. After 14 days, the immunized groups received a booster immunization of 0.2 μg of protein in adjuvant and the mock-immunized groups were injected with normal saline in adjuvant.

At 28 days, both the alpha-toxin-immunized and mock-immunized groups were challenged intramuscularly in the hind leg with various amounts of washed *C. septicum* cells. Five groups of 20 mice each, 10 of which were immunized with toxin and 10 of which were mock immunized, were challenged with 10^3 to 10^7 CFU of washed log-phase *C. septicum* organisms in 100 μl of normal saline. The animals were then observed for 72 h, and the course of the infection and time to death were recorded for each animal. Only those groups which exhibited 100% mortality in the mock-immunized controls were used in the final analysis.

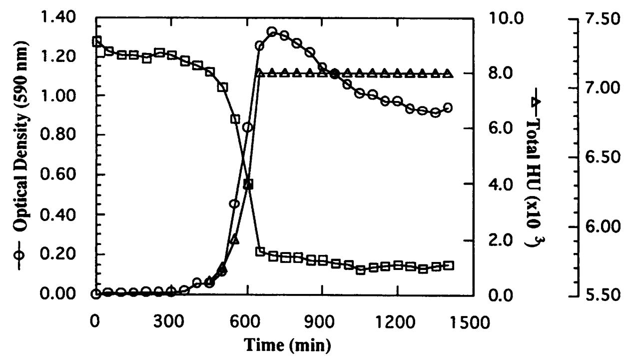

FIG. 1. Growth of *C. septicum* and expression of alpha-toxin. Shown is the growth of *C. septicum* BX96 and the expression of hemolytic activity. More than 95% of the hemolytic activity was attributed to alpha-toxin, and therefore the observed activity reflects primarily alpha-toxin expression during the growth.

RESULTS

Growth of *C. septicum* and purification of alpha-toxin. Initial analysis of the growth of *C. septicum* showed that production of hemolytic activity corresponded with log-phase growth (Fig. 1). It was later confirmed that >95% of the observed hemolytic activity was attributable to the alpha-toxin. The log-phase growth and toxin production occurred over a 2.5-h interval, with alpha-toxin production terminating at the transition period between the log and stationary growth phases. The pH during log growth time dropped dramatically; however, this had no effect on the termination of the alpha-toxin production since maintaining the pH at 7.0 did not change the production pattern of alpha-toxin (data not shown).

The purification of the lethal factors produced by *C. septicum* was followed by a mouse lethality assay since it was unclear whether more than one lethal factor was present. Only the unbound fraction from the first anion-exchange column (Accell QMA) exhibited detectable lethal activity. This indicated that the lethal toxin was a basic protein since it did not bind to this column at pH 8.0. The isoelectric point of the alpha-toxin was later confirmed to be approximately 8.4 by chromatofocusing (data not shown). Throughout the purification, it was found that the lethal activity copurified with the majority of the hemolytic activity. This confirmed the earlier observation of Bernheimer (2)

TABLE 2. Purification table for *C. septicum* alpha-toxin

Step	Vol (ml)	Total HU	Total protein (mg)	HU/mg	% Yield[a]	Purification (fold)
1. Culture supernatant	5,000	2.6×10^7	103,000	246	100	1
2. Concentrate	110	7.2×10^7	5,673	12,707	281	51
3. Accell QMA	220	2.25×10^8	1.287	1.8×10^5	879	711
4. Superose S	2.0	1.6×10^7	16.3	1.0×10^6	64 (14)	4,075
5. Superose 12	1.2	9.8×10^6	2.7	3.7×10^6	38 (4.3)	15,023
6. MA7S	1.0	1.4×10^7	0.7	2.1×10^7	56 (6.3)	84,214
MA7S (aggregated alpha-toxin)[b]	0.4	51,200	0.04	1.3×10^6	NA[c]	NA

[a] The percent yield shown is based on the starting activity in step 1, whereas the numbers in parentheses are the yields based on the activity present in step 3.
[b] The amount of aggregated alpha-toxin is shown for comparison with monomeric alpha-toxin (step 6).
[c] NA, not applicable.

who, in 1944, suggested that the lethal activity and hemolytic activity of *C. septicum* were due to the same molecule.

The overall yield of alpha-toxin was found to be consistently around 50% (Table 2) based on the recovery of hemolytic activity; however, it was closer to 6% based on the increase in activity present after the third step (Accell QMA anion exchange) in the purification. A 48-kDa protein increased in abundance throughout the purification and was shown to be homogeneous by SDS-PAGE (Fig. 2A) and by native gel electrophoresis (Fig. 2B). As can be seen in Table 2, the total hemolytic activity of the preparation increased significantly after the third purification step, which involved the passage of the concentrated extracellular proteins over an anion-exchange column. More than 75% of the contaminating protein bound to the column, whereas alpha-toxin passed through without binding because of its basic isoelectric point. Therefore, a significant amount of the contaminating protein was removed, which may have removed a potential endogenous inhibitor of the alpha-toxin. What was unexpected, yet consistent, in all the purifications was the loss of activity experienced after the cation-exchange chromatography with Superose S. Only about 14% of the total activity from the Accell QMA fraction was recovered. This was probably due to lability of the toxin and the fact that the toxin was kept at 5°C overnight to complete the chromatography. Little loss in activity was found to occur during the next two purification steps, possibly because they took less than 60 min to complete. A missing cofactor did not appear to account for the loss in activity since add-back experiments did not appear to increase the activity of the fractions from the Superose S column and the specific activity of the toxin continued to increase throughout the purification.

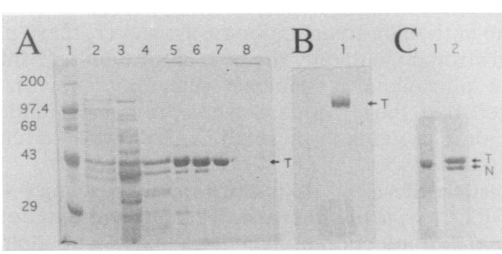

FIG. 2. PAGE analysis of alpha-toxin. (A) Various proteins present in each step of the purification were separated by SDS-PAGE analysis. Lane 1, molecular weight markers (the molecular weight of the markers [$\times 10^3$] is denoted to the left of the gel); lanes 2 to 7, proteins present in the purification fractions after steps 1 to 6, respectively; lane 8, aggregated form of alpha-toxin. The nicked form of the toxin (approximately 44 kDa) can be seen as a faint band immediately below the alpha-toxin band. Its presence was more pronounced in older preparations of the toxin (see panel C) as a result of slow proteolytic nicking. (B) The same amount of alpha-toxin as used in lane 7 of panel A was separated by native acidic PAGE. (C) The same toxin preparation was analyzed at two different times by SDS-PAGE. Lane 1 contains a freshly prepared lethal toxin sample, and lane 2 contains the same toxin preparation after 10 months. The sample had been stored at −20°C and was occasionally thawed to remove samples for various analysis. In all cases, the letter T denotes the intact toxin and the letter N denotes the nicked form.

The purified alpha-toxin was found to be a protein of approximately 48 kDa with a pI of 8.4. The alpha-toxin was found to have an LD_{50} of approximately 10 μg/kg of body weight for mice and exhibited hemolytic activity with a specific activity of approximately 2×10^7 hemolytic units (HU) per mg. The alpha-toxin-induced hemolysis usually took a minimum of 3 to 6 h for complete lysis, and typically the hemolytic assays were allowed to proceed overnight. The phenomenon known as hot/cold lysis, typical of *C. perfringens* alpha-toxin, in which hemolysis is enhanced by incubating the erythrocytes at 4°C after toxin treatment at 37°C, was not observed for *C. septicum* alpha-toxin.

A smaller protein of about 44 kDa was found to be present in most lethal toxin preparations to variable degrees (Fig. 2C). This smaller protein appeared to be derived by proteolytic cleavage from the 48-kDa lethal toxin since with time its abundance increased in purified preparations of the toxin. It was also immunoreactive with antibody affinity purified to the 48-kDa lethal toxin (see Fig. 4), and peptide maps of the 48-kDa toxin and the 44-kDa protein were similar (data not shown).

The hemolytic activity found associated with the lethal fraction was distinct from at least two other hemolytic activities which were found in fractions that did not contain detectable lethal activity. One of the hemolytically active fractions from the Accell QMA column eluted between 50 and 100 mM NaCl and was most likely the thiol-activated cytolysin, septicolysin (delta-toxin), which is presumably produced by *C. septicum* (7). This hemolysin accounted for less than 5% of the total detectable hemolytic activity. A second hemolytic fraction which contained a protein of approximately 230 kDa was identified at the final purification step (MA7S cation-exchange resin). This fraction also contained minor amounts of the monomeric lethal toxin and nicked form. Subsequent immunoblot analysis showed that the 230-kDa protein was an aggregated form of the alpha-toxin. This aggregated form was largely resistant to dissoci-

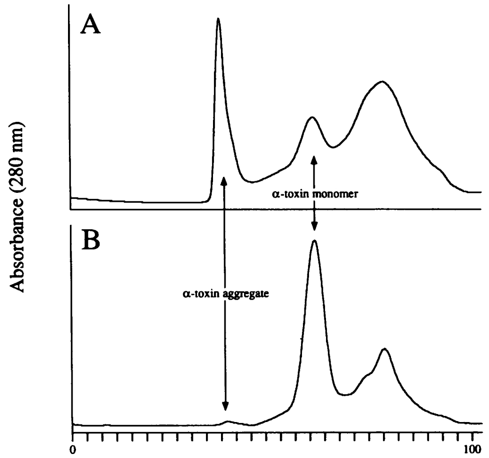

FIG. 3. Concentration-dependent aggregation of alpha-toxin. The appearance of the 230-kDa aggregate of alpha-toxin was shown to be concentration dependent by passing alpha-toxin obtained from step 3 (Superose S) of the purification scheme over a Superose 12 gel filtration column. (A) Concentration of the alpha-toxin fraction was 8 mg/ml; (B) concentration was maintained at 2 mg/ml. The relative retention times of the aggregated alpha-toxin and monomeric alpha-toxin are shown.

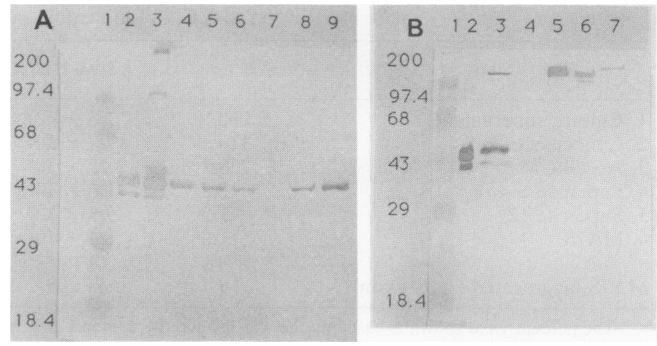

FIG. 4. Presence of alpha-toxin in various *C. septicum* strains and other clostridial species. The proteins in the spent medium (brain heart infusion) from the various *C. septicum* strains or clostridial species were separated by SDS-PAGE and then transferred to nitrocellulose. The immunoblot was then probed with affinity-purified rabbit anti-alpha-toxin IgG. (A) Lane 1, molecular weight markers ($\times 10^3$); lane 2, purified alpha-toxin; lanes 3 to 9, culture supernatant proteins from *C. septicum* BX96, 23, 11424, 25, 8065, 87-016055, and 12464, respectively. See Table 1 for the origin of the various strains. (B) Lane 1, molecular weight markers ($\times 10^3$); lane 2, pure *C. septicum* alpha-toxin; lanes 3 to 7, culture supernatants from *C. septicum* BX96, *C. chauvoei* 10092, *C. histolyticum* 19401, *C. perfringens* 13124, and *C. difficile* 10463, respectively.

ation with SDS in the presence of a thiol reagent and heat (100°C). Also, the addition of approximately 8 M urea with the SDS and thiol reagent had no dissociating effect on the aggregate (data not shown). Both alpha-toxin and the 230-kDa protein reacted on an immunoblot independent of whether the antibody was affinity purified to alpha-toxin or the 230-kDa protein. The appearance of the 230-kDa protein also appeared to be related to the concentration of the lethal toxin. When the lethal toxin-containing fractions from the Superose S column (Table 2, step 4) were pooled and maintained at a concentration of less than 2 mg/ml, the 230-kDa protein was present at low levels; however, concentration of this fraction to approximately 8 mg/ml induced the formation of the 230-kDa aggregate of alpha-toxin (Fig. 3). Although it is clear that the toxin and/or its nicked form can aggregate, it is unclear whether this is related to the hemolytic mechanism of the toxin. The hemolytic activity of this fraction was found to be only about 10 to 20% of that of the monomeric toxin on a weight basis.

Occurrence of alpha-toxin in various *C. septicum* strains and other clostridial species. Spent medium from seven *C. septicum* strains was separated by SDS-PAGE, transferred to nitrocellulose, and probed with the anti-alpha-toxin antiserum (Fig. 4A). Immunologically cross-reactive bands that comigrated with purified alpha-toxin were detected in all strains of *C. septicum*. Two additional immunoreactive bands were also present in the *C. septicum* BX96 culture filtrate. The first is an IgG-binding protein which migrates close to the 97.4-kDa marker. This protein was recognized by the goat anti-rabbit IgG-alkaline phosphatase conjugate in the absence of the affinity-purified antibody (data not shown) and is apparently a previously undescribed IgG-binding protein. The second protein is the high-molecular-weight aggregate of the alpha-toxin as described above. The appearance of the aggregated form was variable; in the culture filtrate of strain BX96 in Fig. 4A, it was present, but in Fig. 4B it was not present. The lack of the aggregated form of the toxin in the other strains of *C. septicum* may be the result of a lower production of the toxin than that found in BX96. Strain 8065 appeared to produce considerably less alpha-toxin than the other strains; however, a faint band which comigrated with alpha-toxin was consistently detected on the original immunoblots, although it is not as obvious on the photographic reproduction in Fig. 4A. An immunoblot of the crude culture supernatant fluid from *C. perfringens*, *Clostridium histolyticum*, *Clostridium difficile*, and *Clostridium chauvoei* was also probed with anti-alpha-toxin affinity-purified antibody (Fig. 4B). This immunoblot did not reveal any cross-reactive proteins present in these strains that were similar in size to the alpha-toxin. However, the blot did reveal high-molecular-weight proteins in *C. perfringens*, *C. difficile*, and *C. histolyticum* which appeared to cross-react with the anti-alpha-toxin antibody. However, these proteins reacted strongly with only the goat anti-rabbit IgG antibody-alkaline phosphatase conjugate (data not shown), which indicated that these proteins were previously unidentified IgG-binding proteins in these strains and were unrelated to alpha-toxin.

Protective effects in alpha-toxin-immunized mice to challenge with *C. septicum* infection. The survival time following a *C. septicum* infection of mice immunized with alpha-toxin showed a significant increase when they were compared with a mock-immunized group. In Fig. 5, the survival times of the immunized and mock-immunized groups are shown after challenge with live, washed *C. septicum* cells. At 17 h postchallenge, there was a 70% mortality in the mock-immunized group, while the immunized group showed only a 20% mortality. Overall, the mock-immunized group exhibited no survivors after 24 h when challenged with *C. septicum*, whereas the immunized group exhibited a 30% survival.

DISCUSSION

The *C. septicum* lethal toxin was purified and found to be the only detectable lethal factor present in crude culture supernatants of *C. septicum*, which was consistent with the

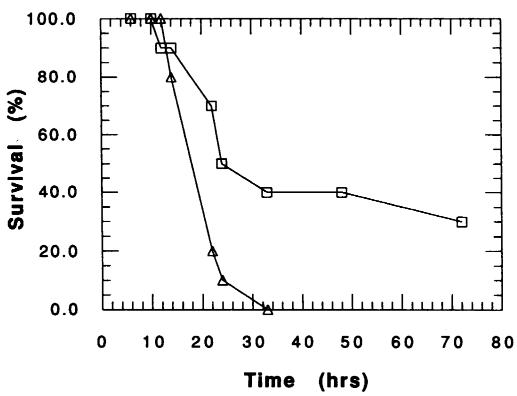

FIG. 5. Survival time of mice immunized with alpha-toxin. The survival times for mice immunized with alpha-toxin (□) versus those immunized with adjuvant only (△) are shown. Mice in the immunized group that survived to 70 h completely recovered from the *C. septicum* infection.

early suggestion of Bernheimer (2). The lethal toxin was found to be a basic protein with an isoelectric point of about 8.4. The hemolytic activity for the purified alpha-toxin was approximately 2×10^7 HU/mg, and the LD_{50} was approximately 10 μg/kg of body weight for mice. The hemolytic activity of this toxin was similar to that found for some of the most potent hemolysins, such as *C. perfringens* theta-toxin (22). Overall yield of alpha-toxin was 56%, based on the activity found in the first step of the purification; however, if the final yield was based on the activity present after the third step in the purification, the yield was closer to 6%. Regardless of the yield, the specific activity of the toxin increased throughout the purification, indicating that the toxin preparation did not contain an increasing amount of inactive toxin due to the loss of a cofactor. The explanation for the unexpected rise and then fall of the total hemolytic units in steps 3 and 4 of the fractionation remains unclear. It is likely that the first chromatographic step removed an endogenous inhibitor that resulted in the increased activity. The loss at the next chromatographic step may be due to the lability of the toxin, which has been observed previously in crude preparations (19). Inactivation of the toxin may occur by proteolytic cleavage since it is clearly susceptible to one or more proteases produced by *C. septicum*. Alternatively, the fact that the toxin readily undergoes a concentration-dependent solution aggregation may account for the observed loss in the activity since the aggregate is considerably less active than the monomer.

Purified toxin appeared to undergo proteolytic nicking over time that resulted in a slightly smaller form of 44 kDa. The significance of the smaller form of the toxin is currently unclear since it does not appear to affect the total activity of the toxin preparation. Another curious aspect of the toxin is the fact that it undergoes a concentration-dependent solution aggregation. This aggregate was found to be resistant to denaturation by SDS in the presence of a thiol reagent and heating. Based on its apparent mass, it appears to be made up of five to six monomeric subunits; however, the estimation of the mass of the aggregated form may be inaccurate since it is outside the range of the molecular weight markers. Interestingly, the concentration-dependent solution aggregation of the alpha-toxin resembles that described for the pore-forming hemolysin (aerolysin) from *Aeromonas hydrophila* (9).

Immunoblot analysis of other *C. septicum* isolates with the anti-alpha-toxin antibody confirmed the presence of the alpha-toxin in strains other than BX96. More recently, several other *C. septicum* clinical isolates have been screened by immunoblot analysis and were found to express alpha-toxin (23). All *C. septicum* strains examined thus far have been found to produce an immunoreactive protein that comigrates with the purified alpha-toxin, although at least one strain (strain 8065, Table 1 and Fig. 4) appears to produce significantly less alpha-toxin in culture. *C. perfringens*, *C. histolyticum*, *C. chauvoei*, and *C. difficile* were not found to produce an extracellular protein that exhibited immunological relatedness to alpha-toxin. The only antibody-reactive proteins present in these strains turned out to be previously uncharacterized IgG-binding proteins in *C. perfringens*, *C. difficile*, and *C. histolyticum*. It appears that IgG-binding proteins may be common among the pathogenic clostridia. Somewhat of a surprise was the lack of cross-reactive proteins from *C. chauvoei* and *C. histolyticum* since earlier studies suggested a relationship between the lethal factor produced by *C. septicum* and those of these two species (19, 20). Since these early studies were performed with complex protein mixtures as the immunizing antigens, the results may not have reflected the true relationship between the toxins of *C. septicum* and *C. chauvoei* or *C. histolyticum*. However, it is also possible that conserved epitopes between the *C. septicum* alpha-toxin and the uncharacterized lethal toxins of *C. chauvoei* and *C. histolyticum* were sensitive to sample denaturation for SDS-PAGE.

Mouse protection studies were used to determine whether immunization of mice with the lethal toxin afforded any degree of protection after challenge with live *C. septicum* organisms. Mice were chosen since they have been reported to be quite susceptible to *C. septicum* infection (19). Mock-immunized animals demonstrated a significantly shorter time to death when compared with an immunized group. A precipitous increase in mortality was observed in the mock-immunized group between 14 and 17 h. At 17 h following challenge, only 20% of the mock-immunized animals survived, whereas 70% of the immunized animals survived. These results indicated that immunization with the alpha-toxin afforded the mice at least partial protection against challenge with *C. septicum*.

Several questions remain concerning the significance of the nicked form of the toxin and the high-molecular-weight aggregate of the toxin in relationship to the mechanism of action of alpha-toxin. It is clear that this toxin does not exhibit a mechanism similar to that of *C. perfringens* alpha-toxin (i.e., phospholipase C and sphingomyelinase C activity) since the lack of such activity is a hallmark of *C. septicum* and is often used to differentiate *C. septicum* from *C. perfringens*. The *C. septicum* alpha-toxin also does not appear to exhibit phospholipase D or sphingomylinase D activity (24). The fact that it appears to form SDS-resistant aggregates in solution may indicate that it is a pore-forming hemolysin; however, this remains to be investigated.

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